

Increasing of Macrophage Migration Inhibitory Factor Expression in Human Patients Infected with Virulent *Brucella* in Iraq

Hasan R. Khudhur^{1*}, Abbas Ali Menshed², and Ahmed Abbas Hasan³

¹Department of Medical Microbiology, AL-Muthanna University, 66001, Iraq

²Ministry of Education, ³College of Health and Medical Techniques, Al-Furat Al-Awsat Technical University, 31003, Iraq

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Brucellosis is a zoonotic disease caused by *Brucella* infections and humans usually contract this disease from close contact with infected animals or their products, usually via the ingestion of cheese or crude milk. Macrophage migration inhibitory factor (MIF) and Pro- and anti-inflammatory cytokines play an important role in susceptibility/resistance and the immunopathogenesis of *Brucella* infection. These cytokines are crucial factors in the initiation and progression of protective immunity against *Brucella* infection but the role of MIF has not been well studied in the human response to intracellular microbes. This study was designed to investigate the effect of MIF expression on *Brucella* susceptibility. A total of 85 positive rose Bengal tests and 24 samples from healthy individuals were collected for this study and subjected to polymerase chain reaction assays (PCR) of the *bcsp31* diagnostic gene. MIF concentrations were evaluated using Enzyme-Linked immunosorbent assay (ELISA) and the results showed that 46 (54%) of the rose Bengal test samples were positive and 39 (46%) were negative for *bcsp31* ($p \leq 0.05$) and used as the gold standard for all of the comparisons in this study. The ELISA results indicate that the mean concentration of MIF was significantly higher in patients with positive rose Bengal tests when compared to the control groups and that its concentration increases with increasing age in both the patient and control groups ($p \leq 0.05$).

Keywords: Brucellosis, Iraq, *bcsp31*, MIF, cytokine

Introduction

Brucellosis is a well-known infectious disease that affects humans and many domesticated animals. This disease is known to be a global problem and one of the most important and dangerous zoonoses diseases in central and South America, India and Mediterranean region [1]. Brucellosis is caused by *Brucella* bacteria, which don't form flagella, spores, and true capsule, *Brucella* is gram-negative and commonly don't appear bipolar staining, it's not truly acid-fast, but resist decol-

oration by weak acid [2]. The genus *Brucella* is phylogenetically located under phylum - proteobacteria, the group that includes different types of bacteria with different lifestyles [3]. There are six species that can distinguished within the genus *Brucella*: *B. ovis*, *B. abortus*, *B. neotomae*, *B. suis*, *B. melitensis* and *B. canis*. This classification depends on differences shown in, pathogenicity, biochemical characteristics and host preferences [4]. Pathogenesis of *Brucella* separated into four steps as another intracellular microorganism: adherence, invasion, establishment, and dissemination within host tissue [5].

B. abortus suppresses immunity (pro-inflammatory) during the early stages of infection, then followed by mild expression of pro-inflammatory chemokines,

*Corresponding author

Tel: +009647733668314

E-mail: hassanraheem1990@gmail.com

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especially CXCL 6 and CXCL 8 in trophoblastic cells *in vitro* [6]. The acquired immune response to infection by facultative intracellular bacteria, *Brucella* species enhance both humoral and cellular immunities [7]. The *bcs31* gene, coding for a 31-kDa immunogenic outer membrane protein conserved in all species of *Brucella*, the *bcs31* is the common genetic target in the most clinical applications [8]. Such a PCR technique is specific for genus can help to avoid false negative results in patients infected with biovars and unusual species [9].

Neutrophils, macrophages, natural killer cells, and T cells are responsible for the innate immunity in the early stage of infection, and adaptive immunity against *Brucella* depended on B & T cells, lymphokines produce by T cell are involved in attracting immunity cells to the infection center. This leads formation of granuloma, this inflammatory response is supported by cytokines produced by many cell types such as tumor necrosis factor (TNF), the colony-stimulating factor (CSF) and interleukin-1 (IL-1) [10].

MIF is a pleiotropic cytokine synthesized primarily by immune cells, such as neutrophils lymphocytes and dendritic. MIF enhances and supports the production of several inflammatory molecules, such as cyclooxygenase 2 (COX-2), nitric oxide and TNF- α . MIF is also essential in the migration and recruitment of immune cells to expression of chemokines as adhesion molecules as an intercellular adhesion molecule (I-CAM-1), monocyte chemoattractant protein (MCP-1), and vascular cell adhesion molecule as V-CAM-1. MIF have essential role against intracellular pathogen as *Mycobacterium tuberculosis*, *Leishmania* spp and brucella [11–13]. *In vivo* study observed that MIF acts as an immunomodulatory effector in the first stages of pulmonary granulomas in mice [14], and there are recent study findings increased levels of MIF in pulmonary tuberculosis patients [15].

Experimental

Subjects and study design

The study was accomplished at Al-Hussein Teaching Hospital in Al-Muthanna Province, Iraq. This province is located in the southeastern region of country.

A total of 46 patients infected with brucellosis (34 male, and 12 female: mean age = 18 ± 13 years old), Patients with brucellosis diagnosed based on isolation,

laboratory tests, clinical, bacteriological parameters as well as PCR technique, *B. abortus* was isolated by Lysis-centrifugation method, and diagnosis of that strain was done by the API-20E system, Vitek 2 System and PCR [16]. Control group (16 males and 8 females: mean age = 20 ± 10 years old), included 24 apparently healthy person without any brucellosis or any disease.

Bacterial genomic DNA

The bacterial genomic DNA of *Brucella* species isolates, were extracted by using Geneaid DNA extraction kit according to the manufacturers' instructions. The PCR was cured out using primer sequence F: 5'-TGGCTCGGTTGCCAATATCAA-3 and R: 5'-CGC-GCTTGCCTTTCAGGTCTG-3 with size product 223 bp [17]. The Mixture volume of PCR was 20 μ l, including (1X) Pre master mix (Bioneer, Korea), 1 μ l of each primer (10 pm/ μ l), 2.5 μ l of genomic DNA (20 ng/ μ l) and volume were completed with Nuclease free water. PCR conditions were: 95°C for 5 min then 45 cycles of 95°C for 20 sec, 60°C for 30 sec, and 72°C for 40 sec, followed by 10 min extension at 72°C. The products of PCR were processed by electrophoresis in a (1%) agarose gel and stained with (10 mg/ml) ethidium bromide.

Determination of cytokines

Three milliliters (3 ml) of blood were centrifuged at (4700 RPM for 5 m) to obtain serum then cooled at -20°C until the time of the serological test. Serum MIF cytokines levels were determined by the ELISA technique using a quantitative sandwich enzyme immunoassay technique (ELISA kits for MIF by PeproTech Company/Germany). All tests were done according to company instruction. The results calculated by ELISA reader and desinged on a standard curve to processes the concentration of the cytokine.

Statistical analysis

Statistical package for social science (SPSS) 26 were used to analysis of data. All data were presented as mean \pm standard deviation (SD). The differences between the frequencies processed by using A Chi-square test, and mean values between groups was compared by T-test. ANOVA and LSD test were used to find out the significant differences between more than two groups of continuous variables. The statistical significance was

accepted as a p value < 0.05 [18].

Results and Discussion

The results in isolating the bacteria from the human blood in the current study may have belonged to most samples taken during the winter where the presence of few numbers from bacteria in the blood and the use of antibiotics during the sampling.

In the present study 46 (54%) of 85 blood sample was brucella positive. The samples taken from peripheral blood to culture because it is difficult to obtain samples from the lymph nodes or bone marrow or synovial fluid as well as the spinal cord. Many patients also neglecting initial diagnosis to bacteria that turn into chronic phase and then after that is difficult to isolate. the sensitivity of isolation in the acute form had been reported as lower than 90% and in the chronic form, to be as low as 20% [19]. Previous study in Iran not conforming to the current study where they found 8 (26.6) blood culture cases were positive from 30 (100%) cases collecting from peripheral blood, and all 8 strain diagnoses as *B. melitensis* [20]. Other previous study in Macedonia found 16 (14.2%) blood culture cases were positive from 113 (100%) cases collecting from peripheral blood [21]. Al gaiashi In Iraq (2017) found 38 (38%) samples were positive and 62 (62%) were negative from 100 (100%) cases collecting from peripheral blood collecting [22]. PCR assay was developed for the diagnosis of brucellosis, by amplifies the target gene of Brucella species. Primer include for encoding 31-kDa immunogenic *B. abortus* protein (BCSP 31) [17]. The specific diagnosis of diagnostic genes in all studied strains ($n = 46$) showed that all *B. abortus* isolates were positive for BCSP 31 46 (100%). These results were in agreement with [22] in Iraq, which found all *B. abortus* isolates (38 100%) were positive for *bcs31* gene.

Studies found that PCR appeared to be a more sensitive technique than microbiological methods, for the diagnosis of a first stages of infection, and early detection of relapses [23].

Fig. 1 shows that the mean MIF cytokine concentration has a statistical difference between patients and healthy control subjects (3.9626 ± 0.8303 ng/ml) versus (2.0960 ± 0.3782 ng/ml) respectively ($p = 0.050$).

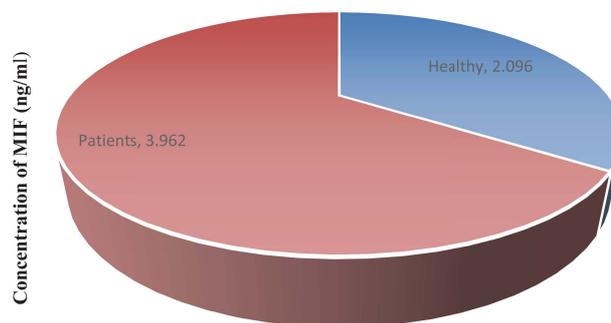


Fig. 1. Comparison of Mean Serum MIF Cytokine Between Control Group and Patient Group (T-test- $p = 0.050$).

Macrophage migration inhibitory factor (MIF) is one of the important first cytokines to be discovered, its consider as an supportive component of the immune response against microbial and induce production and secretion of interleukins like IFN- γ , IL -1 β , IL-6, IL-8, IL-12 and TNF- α by immune cells [24]. MIF increases the survival of macrophage and decrease activation-induced apoptosis by inhibition activity of P53 [25, 26]. There are no previous studies associates with this study, other studies on another disease such as in Switzerl and [27] found that MIF concentration in patients with Tuberculosis more than control groups as well as another study with another disease such [28] in Iraq and [29] in Brazil which they found that MIF concentration in patients with Cutaneous leishmaniasis more than control groups.

Fig. 2 shows that the mean concentration of MIF in patients and control groups according to variation in age was ($p < 0.001$) respectively. It was found that the mean of MIF level was declining continuously with age in patients and healthy control.

Table 1 shows that the mean concentration of MIF levels according to gender in patients and control groups, the mean concentration of MIF was not a significant difference between male and female inpatients and control groups.

The age groups of Brucellosis patients in this study Table 2 were classified into six age groups (11–20 year, 21–30 year, 31–40 years, 41–51 years, 51–60 and above 60 years), patients were included in this study their ages ranging between 10–70 years old, 14 (30.43%), ($p < 0.001$).

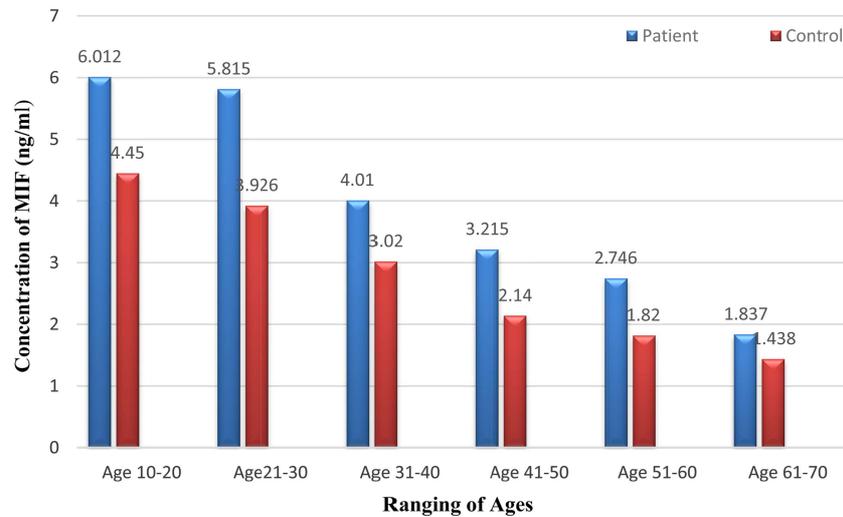


Fig. 2. Correlation between age and serum MIF in patient and control groups (ANOVA and LSD test- $p < 0.001$)

Table 1. Comparison of mean serum MIF Between male and female in patients and control (T-test).

	Male	Female	p -value
Patients	4.45 ± 1.13	3.87 ± 1.22ng	$p = 0.594$
Control	3.67 ± 1.82	3.26 ± 1.98	$p = 0.538$
p value	$p = 0.045$	$p = 0.048$	-

Table 2. Distribution of Brucellosis according to age, (ANOVA and LSD test).

Age	No.	Female	Male	p -value
11-20	8 (17.39%)	6	2	0.157
21-30	6 (13.04%)	6	0	-
31-40	6 (13.04%)	6	0	-
41-50	14 (30.43%)	8	6	0.593
51-60	8 (17.39%)	6	2	0.157
61-70	4 (8.70%)	2	2	-
Total	($p < 0.001$).	34	12	0.001

Conclusions

In conclusion, this study shows that Brucellosis is considered an endemic disease in Iraq, where all age groups are affected. In acute infection of brucella, isolation of bacteria from blood or other tissues is difficult, culture is usually negative, especially in infection with long-standing. PCR assay is specific, highly sensitive, efficient, reproducible method for the rapid and safe detection of

the genus *Brucella*. MIF secreted by activated Macrophage has essential role in innate immune response against Brucellosis in humans.

Conflict of Interests

The authors have no financial conflicts of interest to declare.

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